Crystallization of Prostaglandin-H Synthase for X-Ray Structure Analysis

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Prostaglandin-H (PGH) synthase from ram seminal vesicles is a dimeric integral membrane protein of molecular weight 140 kDa. PGH synthase is a key enzyme in the biosynthesis of prostaglandins, has cyclooxygenase and peroxidase activities, and contains heme as a coenzyme. In the peroxidation step of its reaction, PGH synthase can use xenobiotics as co-substrates and can catalyze the metabolic activation of carcinogens such as diethylstilbestrol. To gain a detailed understanding of the inner workings of PGH synthase, we are investigating its three-dimensional structure by X-ray crystallography. A purification procedure was established that yields stable homogeneous PGH synthase that is at least 80% holoenzyme. The crucial aspect is the proper choice of type and concentration of detergent in all steps of the procedure. Single crystals can be obtained from concentrated solutions of PGH synthase in the presence of polyethylene glycol 4000 as a precipitant. Crystallization occurs during gas phase equilibration with a concentrated salt solution. The enzyme solution becomes turbid and forms a second liquid phase in which PGH synthase crystals grow up to 0.2 mm in length in the course of days. Manipulation of these crystals is very difficult due to the small volume of the growth phase. The crystals dissolved rapidly in all aqueous media into which they were transferred for mounting in X-ray capillaries. Therefore, we have not yet been able to demonstrate their true X-ray scattering power. A crystal provisionally dry mounted diffracted to about 8 Å resolution.

Introduction

Prostaglandin-H (PGH) synthase is an enzyme that holds a key position in the biosynthesis of prostaglandins. Prostaglandins are mediators of many physiological and pathophysiological processes such as inflammation. The importance of PGH synthase-dependent reactions is underlined by the fact that many drugs have been developed that are inhibitors of PGH synthase (e.g., aspirin).

PGH synthase has cyclooxygenase and peroxidase activities and contains heme as a coenzyme. In the peroxidation step of its reaction, PGH synthase can oxidize endogenous compounds as well as xenobiotics as co-substrates. PGH synthase thus is assumed to play a role similar to that of cytochrome P-450 in the metabolic activation of carcinogens (e.g., diethylstilbestrol). More information about the metabolic role of PGH synthase and its reactions is given by Marnett (1).

To gain a better understanding (and possibly control) of the inner workings of PGH synthase, we are investigating its spatial structure by X-ray crystallography.

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The final goal of this project is to lay the structural foundation for molecular modeling, for developing better drugs, and, possibly, for developing inhibitors of unwanted reactions of PGH synthase. Three-dimensional structure determination of membrane proteins has come within reach in recent years with the availability of new nonionic detergents which allow for improved protein purification and crystallization (2,3).

Properties of PGH Synthase

Enzyme isolated from ram seminal vesicles is the PGH synthase species most widely investigated due to its relatively good yield and reasonably uncomplicated purification. PGH synthase is an integral membrane protein, a homodimer of molecular weight 140 kDa. The enzyme contains 3.5% carbohydrate of the high mannose type (4). The amino acid sequence of PGH synthase has been determined from its cDNA sequence (5–7). The open reading frame codes for 600 amino acids; the N-terminal 24 represent a signal peptide, while the C-terminal 576 constitute the mature protein (Fig. 1).

Serin[506] is part of the active center of PGH synthase; it is acetylated by the inhibitor aspirin (8). Rather than showing a distinct pattern of fatty patches, transmembrane spanning stretches of purely hydrophobic amino acids that would be expected of an integral membrane protein, the mature protein contains only one long sequence of continuously high hydrophobicity, Val[267]–Leu[282] (Fig. 2). It is proposed that the protein consists

Signal peptide:

         − 24   − 1

                M S R Q S I S L R F P L L L L L L S P S P V F S

Mature enzyme:

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A D P G A P A P V N P C C Y Y P C Q H Q G I C V R F G L D R Y Q C D C T R T G Y   40
S G P N C T I P E I W T W L R T T L R P S P S F I H F L L T H G R W L W D F V N   80
A T F I R D T L M R L V L T V R S N L I P S P P T Y N I A H D Y I S W E S F S N   120
V S Y Y T R I L P S V P R D C P T P M G T K G K K Q L P D A E F L S R R F L L R   160
R K F I P D P Q G T N L M F A F F A Q H F T H Q F F K T S G K M G P G F T K A L   200
G H G V D L G H I Y G D N L E R Q Y Q L R L F K D G K L K Y Q M L N G E V Y P P   240
S V E E A P V L M H Y P R G I P P Q S Q M A V G Q E V F G L L P G L M L Y A T I   280
W L R E H N R V C D L L K A E H P T W G D E Q L F Q T A R L I L I G E T I K I V   320
I E E Y V Q Q L S G Y F L Q L K F D P E L L F G A Q F Q Y R N R I A M E F N Q L   360
Y H W H P L M P D S F R V G P Q D Y S Y E Q F L F N T S M L V D Y G V E A L V D   400
A F S R Q P A G R I G G G R N I D H H I L H V A V D V I K E S R V L R L Q P F N   440
E Y R K R F G M K P Y T S F Q E L T G E K E M A A E L E E L Y G D I D A L E F Y   480
P G L L L E K C H P N S I F G E S M I E M G A P F S L K G L L G N P I C S P E Y   520
W K A S T F G G E V G F N L V K T A T L K K L V C L N T K T C P Y V S F H V P D   560
P R Q E D R P G V E R P P T E L                                                   576
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Asn[44], Asn[80], Asn[120], Asn[386]: possible glycosylation sites
Val[267]–Leu[282]: possible transmembrane segment
Ser[506]: aspirin acetylation site

FIGURE 1. Amino acid sequence of ram PGH synthase. From Yokoyama et al. (7).
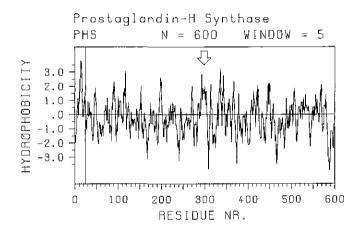

FIGURE 2. Hydropathy plot of the full polypeptide chain of PGH synthase. The signal peptide is separated from the mature protein at residue 24 (shown by a vertical line); the likely transmembrane domain link is marked by an arrow. Coefficients according to Kyte and Doolittle (9) and local FORTRAN program HYPLOT.

of essentially two independent domains (6), soluble protein in character, located on either side of the membrane and connected by a single hydrophobic spacer in extended conformation.

Critical Features of PGH Synthase Purification

In dealing with a membrane protein, the crucial aspect during all steps and procedures is the proper choice of type and concentration of detergent. The detergent has to solubilize the enzyme from the membrane and keep it in solution afterwards, but it should not damage it. For PGH synthase, the commonly used solubilizing detergent is Tween 20. We encountered, however, some problems with this detergent: Tween 20 is often contaminated by peroxides capable of damaging the enzyme protein, and it cannot easily be removed by dialysis and is even concentrated by ultrafiltration due to its low critical micelle concentration, which makes the detergent behave like a macromolecule.

In our laboratory, Tween 20 promoted dissociation of the dimeric enzyme into monomers, loss of coenzyme, and a decline of enzymatic activity (10). Heme-free PGH synthase is less stable and has been found to be readily cleaved by trypsin (11). The apoenzyme can be reconstituted to a functional state by addition of hematin; the stoichiometry of heme binding (0.5 or 1.0 heme molecules per subunit) is still a matter of controversy.

Garavito and co-workers reported crystals obtained from Tween 20-purified PGH synthase that was reconstituted by addition of heme (personal communication; 12). However, these crystals were severely disordered and did not diffract to high resolution (Garavito, personal communication). Therefore, to avoid the problems in reconstituting a holo-dimer from partly damaged apo-monomers, we chose a different approach with the objective of purifying the intact holoenzyme.

A purification procedure for PGH synthase is now established that reproducibly yields a stable homogeneous dimeric product that is at least 80% holoenzyme. For solubilization and during purification, the best

Table 1. Purification of ram PGH synthase.[a]

	Total units[b]	Protein, mg[c]	U/mg	Relative yield, %
Microsomes	605,848	186.3	3,252	100.0
Solubilizate	856,818	81.0	10,578	141.4
DEAE-filtrate	344,545	71.9	4,792	56.9
DEAE-chromatography	243,898	7.8	31,269	40.3
Gel-filtration	234,971	6.3	37,297	38.8
Reconstituted (heme)	243,552[d]		38,659[d]	

[a] PGH synthase from 22 g of tissue.

[b] Enzyme activities are calculated from initial rates of oxygen consumption measured upon addition of 70 μM arachidonic acid to an incubation of PGH synthase in buffer containing 1.0 mM adrenalin according to Kulmacz and Lands (13). Enzyme units are nmole O_2/min.

[c] Protein concentration was determined by the method of Bradford (14) with a Bio-Rad Assay Kit using γ-globulin standard.

[d] Hematin, 1 μM, added to assay incubation mixture described in footnote b.

detergent was N,N-dimethyl-decylamine-N-oxide (DDAO), which replaces Tween 20. For details see Jahnke et al. (10). An example of an enzyme preparation is shown in Table 1.

The apparent increase in enzyme activity upon solubilization of PGH synthase from microsomal membranes is reproducible. The yield of enzyme protein in the subsequent steps compares favorably with published procedures. This yield can be attributed to an optimized purification scheme and to the high quality of the (fresh) tissue: As found by immunoblot analysis, PGH synthase represents more than 10% of the microsomal protein from ram seminal vesicles (G. H. Degen and B. Fischer, unpublished). The final fraction yields a single homogeneous band in silver-stained gels of both native and SDS-PAGE (data not shown).

Preparations different from the one shown in Table 1, when assayed for activity both in the absence and presence of hematin over a period of days and weeks, indicate the presence of probably three different species of PGH synthase molecules: a) native holo-PGH synthase that is rather stable (enzymatic activity assayed without hematin addition); b) native apo-PGH synthase that is initially reconstitutable (determined by an increase in enzymatic activity upon addition of hematin) and which, however, degrades with time to c) damaged PGH synthase. The last species can no longer be reconstituted by heme addition and gives rise to additional bands in SDS gels.

Attention is drawn to the capacity for reconstitution since it may affect the potential of a given enzyme preparation for crystallization in general and the quality of the crystals in particular. PGH synthase can usually be crystallized if the fraction of holoenzyme exceeds 75%. The presence of a limited amount of apoenzyme is a less serious problem than the process of reconstitution itself. We have been able to grow crystals from PGH synthase preparations that contained heme-reconstituted enzyme. These crystals were highly disordered, became insoluble after some days (this effect was also reported by M. Garavito, personal communication) and did not diffract X-rays.

Crystallization

Crystallization of holo-PGH synthase is possible in the presence of the detergent octyl-β-glucoside. Single crystals of native holo-PGH synthase can be obtained from concentrated (> 5 mg/mL) solutions of (nonreconstituted) enzyme in the presence of polyethylene glycol (PEG) 4000 as a precipitating agent. The enzyme solution is equilibrated through the gas phase (hanging or sitting drop method) with a concentrated salt solution.

The enzyme solution concentrated in this way becomes turbid, and mixed micelles are formed that slowly coalesce into a second liquid phase. In this phase brownish PGH synthase crystals grow up to 0.2 mm in length and up to 0.1 mm in width within a few days. A typical crystallization experiment might be started from a solution containing 7 mg/mL holo-PGH synthase and 3.0% PEG 4000 equilibrated with 2.0 M ammonium sulfate (increased stepwise from 1.4 M).

PGH synthase crystals are often in the shape of square prisms. They have sharp edges and faces and display good extinction under polarized light. Unfortunately, their manipulation is very difficult due to the extremely small volume of the growth phase. So far, we have not been able to demonstrate the true X-ray scattering power of the crystals, because all crystals dissolved rapidly in any aqueous medium into which they were transferred for mounting in X-ray capillaries.

At present we are attempting to simulate as closely as possible the second liquid phase in which the crystals grow. The major problem is that the contribution of solute PGH synthase in the solid/solute equilibrium has to be mimicked by substances which are in better supply than PGH synthase itself and yet do not interfere with the crystals.

Nevertheless, we have ventured to mount a crystal dry, accepting a very high risk of damage and fast decay. The dry-mounted crystal displayed reflections out to a resolution limit of about 8 Å in a still photograph. The crystallographic space group could not be determined with the rapidly decaying dry crystal. We are confident that crystals properly mounted in a suitable mother liquor environment will give better results.

NOTE ADDED IN PROOF: The crystallographic space group of the crystals could recently be determined as I 422 with cell constants a = 142Å and c = 172Å. There is one protein subunit in the crystallographic asymmetric unit. Therefore, in the crystalline state, the dimeric molecule is fully symmetrical.

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